PhD Synopsis On

PHYTOCHEMICAL AND PHARMACOLOGICAL EVALUATION OF *MORINDA TINCTORIA* ROXB. AND *MORINDA CITRIFOLIA* EXTRACT AND THEIR SIGNIFICANCE AS ANTI-INFLAMMATORY AGENT

"यत्र नार्यस्तु पूज्यन्ते रमन्ते तत्र देवता:"

JAYOTI VIDYAPEETH WOMEN'S UNIVERSITY (JVWU)

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Ph.D (Pharma)

PHYTOCHEMICAL AND PHARMACOLOGICAL EVALUATION OF *MORINDA TINCTORIA* ROXB. AND *MORINDA CITRIFOLIA* EXTRACT AND THEIR SIGNIFICANCE AS ANTI-INFLAMMATORY AGENT

WORK PLAN AND METHODOLOGY

1) Review of Literature
2) Identification, collection and authentication of the plant
3) Processing of plant materials
4) Preparation of plant extracts of stems and fruits.
5) Isolation of iridoid glycoside from the extracts
6) Animal Ethical Clearance
7) Anti-inflammatoryactivity of ethanolic extract of *Morinda tinctoria* Roxb. and *Morinda citrifolia.*
8) Estimation of biochemical parameters, antioxidant parameters and inflammatory parameters.
9) Comparative study of anti-inflammatory activity of *Morinda tinctoria* Roxb. and *Morinda citrifolia* for effective treatment.

GENERAL INTRODUCTION

In the current scenario, the medicinal plants are much more popular due to the bioactive compounds present in different parts of the herb. These herbs help in preventing the critical diseases in the majority of population in order to promote improved and better health[1]. However, since time immemorial humans are using medicinal herbs, marine organisms, microorganisms, and animal extracts for curing and alleviating severe diseases.

The traditional systems of medicine (TSM) are of great importance as they use natural products as treatment regimen like herbal drugs, micro-enzymes, etc. These medicinal systems include, Ayurveda, Unani, traditional Chinese medicine (TCM), traditional Korean medicine (TKM), and Kampo. However, since thousands and hundreds of years, all of the systems have been in practice throughout the world and have also been flourishing in a well-organized and regulated system of medicine[2].

As per the current classification of WHO for herbal medicines, the herbs have been categorized into four different classes according to their origin, evolution and different forms of current usage.

- Indigenous herbal medicines
- Herbal medicines in systems
- Modified herbal medicines
- Imported products with a herbal medicine base

Indigenous herbal medicines are the one which were used locally by the local community individuals or people belonging to a local region. These medicines and their activity, composition, dosages were known well by the local population due to their long usage. **Herbal medicines** were used since ancient times with their concepts and special theories for the treatment, e.g., Ayurveda, Siddha and Unani. **Modified herbal medicines**includes the modified medications with reference to dosage form, dose, mode of administration, methods of preparation, herbal medicinal ingredients, and medical indications. **Imported products with herbal medicine base**includeall imported herbal medicines like, raw materials and

products.These medicines should be marked and registered in the countries of origin. However, the data of the safety and efficacy must be recorded and submitted to the national authority of the country who imports the medicines[3].

Inflammation is the first response of the immune system to infection or irritation, and plays important pathological roles in the development of diseases such as cancer, diabetes, atherosclerosis, and arthritis. In inflammation, macrophages have three major functions: antigen presentation, phagocytosis, and immunomodulation through the production of various cytokines and growth factors. Macrophages produce various pro-inflammatory cytokines and inflammatory molecules that play a crucial role during the inflammatory process[4]. There are various components to an inflammatory reaction that can contribute to the associate symptoms and tissue injury. Edema formation, leukocyte infiltration and granuloma formation represents such components of inflammation. Edema formation in the paw is the result of a synergism between various inflammatory mediators that increase vascular permeability or the mediators that increase blood flow[5].

OBJECTIVE

In current scenario, herbal drugs and their constituent derivatives is the prerequisite demand in every developing and developed countries due to their medicinal properties and economic procurement. Nevertheless, in the past years, phytopharmacological and phytochemical sciences have determined the new constituents and their pharmacological activities from different plant extracts in order to promote better and improved health benefits.

Morinda tinctoria Roxb. and *Morinda citrifolia* (Family: Rubiaceae) have been used since ancient times in the traditional medicinal systems like, Ayurveda, Siddha, Chinese and many other system of medicines in the treatment of various ailments like, burns, malaria, headache, wounds, cough, cold, liver diseases, hypertension, tuberculosis,skin infections, intestinal worms, diabetes, pain, menstrual disorder, loss of appetite, hernias, urinary tract infection, cancer, cardiovascular diseases, blood pressure, arthritis etc. Seeds and its oil is also used topically on the scalp as insecticide and in the treatment of arthritis. In case of insect stings, flowers are applied topically. The fruits of Noni in Hawaii have been used topically in various conditions like, sprains, swellings, wounds and bruises[6].

Morinda citrifolia (Family: Rubiaceae) contains many phyto-constituents, like, scopoletin, octanoic acid, vitamin C, vitamin A, alkaloids, terpenoids, potassium, anthraquinones (such as nordamnacanthal, morindone, rubiadin, rubiadin-1-methyl ether, anthraquinone glycoside), ß-sitosterol, alizarin, amino acids, L-asperuloside, carotene, caproic acid, caprylic acid, rutin, flavone glycosides, linoleic acid, ursolic acid, and a putative proxeronine. Several flavonol glycosides have also been identified, including an iridoid glycoside from the noni leaves, a trisaccharide fatty acid ester, rutin, two novel glycosides, citrifolinoside, and an asperulosidic acid from the fruit. Six anthraquinones, including 5,15-O-dimethylmorindol, and three iridoids, including morindacin, have also been isolated from *Morinda citrifolia* fruits[7].

It also contains compounds such as, morindacin (iridoid), 5,15-O-dimethylmorindol (anthraquinone)[8], americanin A, asperuloside, narcissoside borreriagenin, citrifolinin B epimer a, citrifolinin B epimer b, asperulosidic acid cytidine, deacetylasperuloside, dehydromethoxygaertneroside, epi-dihydrocornin, D-glucose, D-mannitol, methyl R-D-

fructofuranoside, methyl α-D-fructofuranoside, nicotifloroside, and α -sitosterol 3-O- α -D-glucopyranoside[9].

Morinda tinctoria (Family: Rubiaceae) contains many phyto-constituents, such as, alkaloids, phytosterols, flavonoids, phenols, triterpines, saponins, carbohydrate, tannins, fat and oils[9].

The main objective of the research study is as follows:

1) To collect and processing of selected plant.
2) To study Pharmacognostical characters of stems of *Morinda citrifolia* and leaves of *Morinda tinctoria*.
3) To study the toxicity of plant extracts.
4) To extract active phytoconstituents from stems of *Morinda citrifolia* and leaves of *Morinda tinctoria* by using solvents of different polarities.
5) To identify and isolate active phytoconstituents from stems and leaves by chemical tests, TLC, HPTLC and column chromatography.
6) To characterize the isolated compounds by using Spectroscopical techniques.
7) To screen for anti-inflammatory activity of plant extracts in Wistar rats.

REVIEW OF LITERATURE

Morinda citrifolia

From the fruits of Morinda citrifolia L., one new anthraquinone, 5,15-O-dimethylmorindol,together with five known anthraquinones and one new iridoid, morindacin, together with two known iridoids, were isolated.Their structures were elucidated by analysis of spectroscopic data.[10]

Nagalingam*et al.,* (2012) investigated the phytochemical screening of three different extract of Morinda citrifolia Linn. Phytochemical investigation revealed that *Morinda citrifolia* contains broad spectrum of secondary metabolites along with alkaloids, saponins and reducing sugar in all the three tested extracts.Steroid, phenol, tannin and terpenoids, cardiac glycoside, carbohydrate and flavonoids were also detected in aqueous extract of the fruit. Aqueous and ethanolic extract contains protein content.[11]

Setyani*et al.*,(2018), studied the anti-melanogenesis activity of *Morinda citrifolia* L. (noni) leaves extract to be used in sunscreen. Active constituents like, flavonoids, alkaloids, terpenoids, sterols, and minerals were isolated from the leaves extract of noni. The study showed that the noni leaves extract possessed flavonoid and steroid content with the concentration of 2.82% and 0.14%, respectively[12].

Kochuthressia*et al.,* (2015) demonstrated the extraction and phytochemical screening of *Morinda citrifolia* leaf. Phytochemical screening of two different extract of *Morinda citrifolia* Linn Rubiaceae was investigated. The leaves were extracted in different solvents like, ethanol and water. However, the phytochemical analysis of *Morinda citrifolia* indicated the presence of all major phytochemicals of, alkaloids, carbohydrates, saponins, phytosterols, flavanoids , phenols, proteins, amino acids and a broad spectrum of secondary metabolites[13].

Muenmuang*et al.,* (2017) in this study analyzed the chemical profiling of *M. citrifolia* extract to determine the potential use of *M. citrifolia* by the help of solvent and soxhlet extraction method. Various types of solvents, like, methanol, ethanol and hexane were used to differentially isolate the active compounds of which methanolic extract produced maximum extraction yield. It was

concluded that *M. citrifolia* is the major source of vitamin E, scopoletin, and phenolic compounds indicating its potential to be used in pharmaceutical and food inductries.[14]

Alwala1*et al.,* (2014) aimed to synthesize the silver nanoparticles of *Morinda citrifolia* L.,(Noni fruit)aqueous and ethanolic extractsusing both the aqueous and ethanolic extracts of *Morinda citrifolia* fresh fruits were assessed by 1mM Silver nitrate solution. The study concluded that *Morinda citrifolia* L.,(extract contains important constituents like alkaloids, glycosides, carbohydrates, flavonoids, terpenes, saponins, phenols, tannins, quinones, celluloses, steroids and gums which interprets its medicinal values.[15]

Bittováa*et al.,* (2015) in this study significant dissimilarity in the content of DAA, citric acid and several phenolic compounds in some samples were discovered.Organic acids, deacetyl asperulosidic acid (DAA) and polyphenolic compounds in various noni (*Morinda citrifolia* L.) products (4 juices, 4 dry fruit powders and 2 capsules with dry fruit powder) were analyzed. Simultaneous analysis of organic acids (malic, lactic, citric and succinic acid) and DAA was done by Reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with a variable wavelength detector (VWD) and electrospray ionization time-of-flight mass spectrometer (ESI-TOF MS).Analysis of polyphenolic compound content (rutin, catechin, quercitrin, kaempferol, gallic acid, caffeic acid and p-coumaric acid) was performed by RP-HPLC method with diode-array detector (DAD). [16]

Shami*et al*., (2015) conducted the study to isolate the anthraquinone fractions from the leaves, fruit and roots of *Morinda citrifolia.* The fruit, leaves and roots of *M. citrifolia*were extracted to isolate anthraquinones which exhibited five for the leaves, six red bands for the fruit, and four for the roots under UV light at wavelength 245 nm and 356 nm. Leaves, fruit, and roots showed strong absorption bands at 3275.52, 3408.83and 3308.88 cm-1 respectively identified by O-H stretching. The C=O group of M. citrifolia fruit were detected at the band 1726.27 cm-1, leaves at 1600.06 cm-1 and roots at 1630.71 cm-1. C-H stretching groups were detected at the bands 2954.74 and 2857.38 cm-1 (fruit), 2928.13 cm-1 (leaf), 2922.31cm-1 and 2852.95 cm-1

(root).However, it was concluded that the isolated anthraquinone had a new source of antimicrobials against pathogenic bacteria and as antioxidant.[17]

Deng et al., (2011) conducted the study todetermine the major iridoid components present in different noni plantsparts, and compare the isolated iridoids in noni fruits collected from various tropical areas all over the world. The isolation was achieved on a C18 column with gradient elution using 0.1% formic acid aqueous formic acid and acetonitrile at 235 nm wavelength. The study resulted in isolation of Deacetylasperulosidic acid (DAA) and asperulosidic acid (AA) as major iridoid. The methanolic extracts of noni fruits obtained from different tropical regions resulted in DAA and AA contents of 13.8–42.9 and 0.7–8.9 mg/g, respectively, with French Polynesia having highest total iridoids and the Dominican Republic containing the lowest total iridoid content. It was concluded that Deacetylasperulosidic acid (DAA) and asperulosidic acid (AA) (iridoids) are found to be present in leaf, root, seed and flower of noni plants, and were identified as the major components in noni fruit.[18]

Morindna Tinctoria

Deepti *et al.,* (2012) conducted the study to evaluate the presence of phytochemical constituents and antimicrobial activity of different leaves extracts of *Morinda tinctoria* Roxb.The results revealed that the extracts of leaf contain a broad spectrum of secondary metabolites including, flavonoids, phenols,alkaloids, phytosterols, and triterpenes as major phytochemicals. Methanol extract was found to be more effective against all the microorganisms. Proteus vulgaris (24mm) was found to be most sensitive organism followed by *Klebsiella pneumonia* (21mm) and *Enterococcus feacelis* (21mm). It was concluded that the different extracts of *M. tinctoria* leaves contain a broad spectrum of secondary metabolites and also exhibit antimicrobial activity against all the tested microorganisms.[19]

Sivakumar*et al.,* (2018) conducted the study for preparation of Morinda tinctoria leaves extracts with the different solvent system and evaluation of the bioactivities. The functional groups of extract were identified by Fourier-transform infrared spectroscopy (FT-IR) analysis and the antimicrobial properties were detected by plate assays. The results revealed that the aqueous, ethanolic and methanolic (AEM) extracts contains quinones, steroids, terpenoids, phenols, glycosides,and tannins. FTIR result revealed that AEM comprises of alkyl halides, 1°, 2° amines,

aromatics, aliphatic amines, alcohols, carboxylic acids, esters,ethers, and alkanes, saturated aliphatic, and phenolic groups. The total antioxidant capacity, reducing power, and hydrogen peroxide scavenging activity observed in AEM were about 86.90±0.36, 78.58±0.13, and 80.33±0.09% respectively.The 1, 1- diphenyl 2-picrylhyorazyl and 2, 2-Azinobis-(3 ethylbenzothiazoline-6-sulfonic acids) assay resultsindicated 85.20±0.50 and 52.41±0.60% of free radical scavenging activity in AEM. The protease activity (44.10±0.26%) and protein degradation(44.38±0.58%) were proscribed by AEM. AEM prevents 69.36±0.20% of cell lysis. Thus, it was concluded that AEM leaves showed no harm and were enriched with potent bioactive principles, which can be used further for improved pharmacological applications.[20]

Rajkumar*et al.,* (2018) investigated the wound healing properties of the aqueous extract of leaves of *Morinda tinctoria* Roxb in rats. The study also revealed information on the qualitative, and quantitative analysis,and purification of phytochemicals present in *M. tinctoria* Roxb. Wound contraction and period of epithelialization was determined. Aqueous leaf extract of *M. tinctoria* Roxb when applied topical showed better healing than orally treated and control groups. The results showed that the efficacy of *M. tinctoria* Roxb aqueous leaf extract as a wound healing agent and can also be used as a therapeutic agent for internal as well as external wounds.[21]

Kolli*et al*., (2015) evaluated the total phenolic content and antioxidant activity of *Morinda tinctoria* leaves. The leaves were extracted in different solvents including, hexane, methanol, chloroform, ethyl acetate and were subjected to column chromatography for the isolation of bioactivemolecules. The total phenolic content was highest in methanol extract (5.30±0.011 µg/mg). Cynarin, a hydroxy cinnamic acid was isolated from chloroform extract; oleuropein, a polyphenolic iridoid was isolated from methanol extract. The 2,2-diphenyl-1-picrylhydrazyl radical scavenging efficacy of hexane extract was found to be significant at higher concentration (500 µg/ml- 91.2±0.05%) and the efficacy was found to be significant at lower for ethyl acetate extract (100 µg/ml - 65.1±0.05%).However, cynarin and oleuropein were reported for the first time from Morinda tinctoria leaves.[22]

METHODOLGY AND WORK PLAN

PREPARATION OF TEST EXTRACTS

Percolation of herbal drugs: The extraction for both the plants was carried out with 50% hydroalcoholic by cold percolation method to avoid damage due to heat. The extract was filtered and concentrated under reduced pressure below 40±1°C using roteva vaccum rotary evaporator to dryness to get a constant weight. The % yield will be calculated and will be stored in −20ºC.

ANALYSIS OF PHYTOCHEMISTRY

Processing of selected plant material: the plant material (stems and fruits) collected from the local market were thoroughly washed with clean water and air dried in shade. The plant materials were then subjected to grinding to form coarse powder of fruits and stems which were later sieved by sieve no 10/44. The plant material was then stored in air tight container to be used further.

Evaluation of physicochemical properties of stems and fruit powder: fruits and stemswere evaluated for organoleptic properties like, color, taste, and odour, moisture content, density, loss on drying, extractive values, ash values, microbial assay and phytochemical testing.

Process for extraction: Powder (1 kg) of both the plant parts (fruits and stems) were refluxed separately in Soxhlet apparatus three times with 5L methanol for 6 hours every time. The extract was later concentrated in rota vapour under vaccum at 20-210^{0}C. The extracts were stored in light resistant and air tight containers with separate labeling.

Physicochemical evaluation of extracts: Test extract will be evaluated physically for organoleptic properties including, color, odour, and Taste; pH, Viscosity and Specific gravity.

Phytochemical screening: Identification of phytoconstituents in the plant material will be carried out by the help of different qualitative and quantitative chemical tests.

Test for amino acids: Ninhydrin test, Xanthoproteic test, Folin’s test and Millon’s test

Test for alkaloids: Dragendroff’s test and Mayer’s test

Test for flavonoid: Alkaline reagent test, NH4OH test, Magnesium turning test and Zinc test

Test for phenolic compounds, gum and mucilage, fixed oils, fats, glycosides, steroids, tannins will also be carried out.

Separation of bioactive iridoid glycoside compound: Separation of specific compounds from more significant extract. The ethanol extract (89 g) of fruits and stems will be dissolved in a MeOH–H2O mixture in different ratios and sequentially will be partitioned using chromatographic method on SiO_2 column chromatography using a solvent system of varying polarity. The iridoid-containing fraction will finally be chromatographed on Sephadex LH-20. Further the fraction will be subjected repeatedly to Rp-18 column chromatography followed by SiO_2 column chromatography.

ANTI-INFLAMMATORY ACTIVITY

SCREENING OF ANTI-INFLAMMATORY ACTIVITY OF EXTRACT

ANIMALS AND MANAGEMENT: Adult female Wistar rats weighing 160 ± 20 g were procured from National Laboratory Animal Center, Central Drug Research Institute (CDRI), Lucknow. They were kept in departmental animal house of the Institute. The animals were housed separately in polypropylene cages for acclimitization at a temperature of (23 ± 2 ºC) and 50–60% relative humidity, with a 12 hr light/dark cycle one week before and during the commencement of the experiment. Animal were kept on standard pellet diet (Dayal animal feed, Unnao, India) and drinking water *ad libitum* throughout the housing period. Administration of the extract and toxicant was done by oral route in the morning session throughout the study period. All experimental procedures involving animals were conducted in accordance with the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA).

STANDARD DRUG: Oxaprozin was used as standard drug. The drug dissolved in1% w/v Na CMC and administered orally using a gavages' needle at a dose 600 mg/kg daily for 28 days

PREPARATION OF EXTRACT SOLUTION: The herbal extractswere dissolved in 1% Na CMC and administered orally to the rats at a dose of 200 mg/kg, 300 mg/kg, 500 mg/kg. A group receiving only vehicle, 1% w/v Na CMC was taken as the normal control group.

EXPERIMENTAL DESIGN

Carrageenan-induced rat paw oedema

The ED50 was determined based on the carrageenan induced oedema. Groups of rats (n = 6) were treated orally with Morinda *tinctoria* and *Morinda citrifolia* crude extract (200 mg/kg, 300 mg/kg, 500 mg/kg) 30 min after the injection of the stimulus (carrageenan 1000 g/paw) into the planter surface of right hind paw. Edema was induced by subcutaneous injection of 100 µl of a 1% solution of lambda carrageenan in 0.9% saline into plantar region of the left hind-paw, as was previously described (Morris 2003,[23] Sterile saline solution (0.9%, 0.1 mL) was injected into the left paw as the control reference for the tested paw. The foot volumes of the animals were determined by plesthymographic method described by Ferreira (1979)[24]The inhibition of inflammation was calculated by measuring the volume difference between the right and left paws in comparison with the control group. ED50 was determined from the curve drawn for the percentage of edema inhibition as a function of the dose (Carvalho et al., 1999)[25]

Rats were divided into 6 groups, six animals in each group. After induction of arthritis the animals were grouped and treated as follows

Group-1 Vehicle control	Treated with Sterile saline solution (0.9%, 1 mL/100g BW)
Group-II Negative control	100 µl of a 1% solution of lambda carrageenan on the left hind paw.
Group-III Positive control	The animal is first administered with carrageenan for a period of 28 days for induction. The standard drug Oxaprozin (600 mg/kg orally) is administered from day 1 to day 28.
Group-IV Test treatment	The animals were induced with carrageenan from day1 to day 28 before test extract (200 mg/kg) was admnistered.
Group-V Test treatment.	The animals were induced with carrageenan from day 1 to day 28 before test extract (300 mg/kg) was admnistered.
Group-VI Test treatment	The animals were induced with carrageenan from

	day 1 to day 28 before test extract (500 mg/kg) was admnistered.

INDUCTION OF INFLAMMATION: Arthritis was induced by a single in trans dermal injection (0.1ml) of Freund's Complete Adjuvant (FCA) containing 1.0 mg dry heat-killed Mycobacterium tuberculosis per millilitre sterile paraffin oil into a pad of the left hind paw of female rats. A 26G needle was used for injection. The swelling paw were periodically examined (up to28 days) in each paw from the ankle using Digital Vernier Caliper.

EVALUATION OF Inflammation

Paw Diameter: Paw thickness was measured by compressing the joint by rotating the screw of micrometer screw gauge till the pain elicited as indicated by squeaking or leg withdrawal. The distance moved by the screw gauge was recorded.

ArthriticScore: The rats were assessed every three days for signs of arthritis between day 1and 21 post CFA using scoring system developed to evaluate the severity if AA. Paws were examined for severity of and loci of Erythema, swelling and indurations using a 5-point scale.

0 = paws with no swelling and focal redness

1 = paws with swelling of finger joints

2 = paws with mild swelling of ankle or wrist joints

3 = paws with severe inflammation of the entire paws

4 = deformity or ankylosis.

After the treatment period, the animals were anaesthetized by Diethyl ether (inhalation) and the blood was collected from retro orbital sinus by using capillary into a centrifuge tube which contains EDTA for haematological parameters and without EDTA for serum biochemical parameters and kept in room temperature for 30 min and then centrifuged for 10min for 10,000 rpm. The following biochemical parameters were assayed.

SERUM BIOCHEMICAL PARAMETERS

C - Reactive protein Detection : The CRP-HS is intended for the quantitative determination of CRP by latex principle enhanced immune turbidimetric assay (ITA).

Kit components : CRP Activation buffer, CRP Latex reagent, CRP calibrator

Procedure: Latex particles coated with antibody specific to human CRP aggregate in the presence of CRP from the sample forming immune complexes. The immune complexes cause a light scattering which is proportional to the concentration of CRP in the serum. The light scattering is measured by reading turbidity (absorbance) at 570nm. The CRP concentration is determined from a calibration curve developed from CRP standards of known concentration.

Estimation of urea: Urea is the nitrogen containing end product of protein catabolism. Increased levels of urea in the blood are called as hyper uraemia or azotaemia.

Procedure

Preparation of test sample: Take 1000µl of reagent-1 and 250µl of reagent-2 in 5ml test tube. To this add, 10µl of serum. Mix well and immediately read the test sample and note down the value

Estimation of uric acid: Uric acid and its salt are end products of the purine metabolism. In gout, the most common complication of hyperuricemia i.e. increased serum levels of uric acid lead to formation of monosodium urate crystals around the joints.

ENZYMATIC ANTI-OXIDANT ACTIVITY ESTIMATION OF CATALASE (CAT)

Procedure: To 1 ml of tissue homogenate 4 ml of hydrogen peroxide and 5 ml of phosphate buffer was added and mixed well. From this, 1 ml of solution was taken and mixed with dichromate acetic acid reagent and allowed to incubate for 30 min at room temperature. The absorbance was measured at 570nm. The activity of catalase was expressed as µmole of H_2O_2 consumed /min/mg protein.

DETERMINATION OF SUPEROXIDE DISMUTASE (SOD)

Procedure: The activity of superoxide dismutase was assayed by the method of Kakkar et al., 1984. 0.5 ml of tissue homogenate was taken in a test tube. To this, 1.5 ml of carbonate buffer and 0.5 ml of 0.1 M EDTA was added and mixed well. 0.4 ml of adrenaline was added to initiate the reaction just before the measurement of optical density at 480 nm in a spectrophotometer. The change in optical density was measured for 3 min at an interval of 30 sec and the antioxidant activity of SOD enzyme was expressed as units/min/mg protein. Change in OD per minute at 50% inhibition to adrenochrome transition by the enzyme was taken as one enzyme unit.

Histological Assessment: After euthanasia at the end of 28th day, the hind paws of all rats were amputated above the knee joint and were fixed in 7.4% formalin solution. The paws were then decalcified using 10% nitric acid, embedded in paraffin and sectioned in a midsagittal plane. The sections of articulation of the tarsal joints were stained with haematoxylin and eosin and were examined microscopically for any bony destruction, presence of mononuclear infiltration and pannus formation.

STATISTICAL ANALYSIS

The analysis of data will be done by using one way ANOVA, followed by Tukey's multiple comparison test by using Graphpad prism 5.0 software (Graphpad, San Diego, CA).